Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-011

Procedure Title:	Determination of Ethanol Concentration in Biomass to Ethanol Fermentation Supernatants by Gas Chromatography

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Date: 5/5/94

ISSUE DATE: 5/5/94   **SUPERSEDES:** n/a

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**Determination of Ethanol Concentration in Biomass to Ethanol
Fermentation Supernatants by Gas Chromatography**

Laboratory Analytical Procedure #011

1   Introduction

1.1   Biomass is thought to be a good substrate for bioconversion to ethanol. This ethanol could be used as a more environmentally benign transportation fuel. Various processes have been developed to convert biomass to ethanol. The efficiency of a process can be determined by measuring the yield of ethanol. Gas Chromatography (GC) is a fast and accurate means of determining volatile components such as ethanol.

2   Scope

2.1   This procedure details a method to prepare biomass to ethanol fermentation supernatants for analysis and quantify their ethanol concentration. This packed column GC method utilizes isopropanol as an internal standard to determine concentration, in g/L, of ethanol in the fermentation supernatant. This method is capable of analyzing fermentation supernatant ethanol concentrations ranging from 1 to 50 g/L.

2.2   This procedure describes an associated QA/QC program to demonstrate that the results comply with the Ethanol Project Quality Assurance Plan. Adherence to the Quality Assurance Plan is assumed for all work associated with this procedure.

3   Terminology

3.1   *Biomass to ethanol fermentation*-A process accomplished by enzymatically breaking down biomass structural polymers to monomeric sugars, and utilizing yeast to ferment these sugars to ethanol. Simultaneous saccharification and fermentation (SSF) is one method used to convert biomass to ethanol.

3.2   *Fermentation supernatant*-The liquified portion of a biomass to ethanol fermentation, including cells and biomass debris. For the purposes of the procedure, this refers to slurry samples prior to preparation for analysis.

3.3 *Analytical sample*-A fermentation supernatant that has been filtered and diluted with internal standard for GC analysis.

4. Significance and Use

4.1 Ethanol is the desired product of the biomass to ethanol fermentation process. Accurate quantitation of ethanol concentration is crucial to the design, assessment, and improvement of the process.

5. Interferences

5.1 Biomass to ethanol fermentation supernatants contain high concentrations of organic and inorganic residue. Much of this residue is not volatile and remains in the injection port leading to fouling and activation. An activated injection port can cause the catalytic breakdown of the analytes of interest, before they are detected, adversely affecting the quantitation.

5.2 The biomass to ethanol fermentation process can produce a wide variety of volatile compounds some of which may co-elute with ethanol or isopropanol. This can adversely affect the quantitation.

6. Apparatus

6.1 *Gas Chromatograph (HP 5890 or equivalent)*-Utilizing a flame ionization detector (FID), removable, deactivated glass, injection port liner, associated regulators, gas lines, and septa.

6.2 *Packed GC Column*-6'x1/8" stainless steel Porapak Q (HP 19001A-Q00 or equivalent).

6.3 *Autoinjector (HP 7673 or equivalent)*-Recommended.

6.4 *Data handling system (HP Chemstation or equivalent)*-Typically computer controlled, but may be an integrator or chart recorder.

6.5 *Centrifuge*.

6.6 *Pan balance*-Readable to 0.01g.

7. Reagents and Materials

7.1   *Isopropanol*-Reagent grade.

7.2   *Ethanol*-200 proof.

7.3   *Ethanol*-Separate source, either a 200 proof ethanol standard from a different manufacturer than (7.2), or a commercially prepared solution of known concentration.

> *Note:* Store isopropanol and ethanol reagents appropriately, protect from evaporation and moisture.

7.4   *UHP nitrogen*-GC carrier gas.

7.5   *UHP hydrogen*-FID fuel.

7.6   *Zero air*-FID oxidant.

7.7   *Water*-Reagent grade.

7.8   *Repeat pipette(s)*-Capable of delivering appropriate volumes.

7.9   *Autosampler vials (2 ml) with associated caps.*

7.10   *Crimper*-If necessary.

7.11   *Capped centrifuge tubes.*

7.12   *0.45 **m**m filter*-Either in line syringe filter unit or insert for centrifuge tube.

7.13   *Assorted class A volumetric pipette and flasks.*

7.14   *Paper towel(s).*

8. ES&H Considerations and Hazards

8.1   Utilize caution when handling compressed gasses (especially compressed hydrogen).

8.2   Ethanol and isopropanol are flammable reagents.

8.3 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

9.   Sampling, Test Specimens and Test Units

9.1 Sample the fermentation so as to collect a representative slurry sample.

9.2 If collecting several samples, chill collected supernatants on ice while continuing to collect more.

9.3 Fermentation slurries are to be centrifuged within one hour of sampling. This can be accomplished by spinning at 6000 RPM for five minutes.

9.4 Filter the liquid portion of the sample through a 0.45 μm filter.

9.5 Dilute each filtered fermentation supernatant into a labeled autosampler vial. For typical fermentation supernatants, a tenfold dilution brings the ethanol concentration in the analytical sample within the linear range of the calibration curve. In this case, 100 μL of filtered fermentation supernatant is added, using a suitable repeat pipette, to 900 μL internal standard spiking solution. If the concentration of the analytical sample falls outside of the linear range, re-dilute the sample accordingly.

9.6 For every 25 analytical samples, prepare a sample in duplicate. Duplicates are used to analyze the method precision.

9.7 For every 25 analytical samples, prepare a method verification standard by pipetting 100 μL intermediate calibration verification solution into 900 μL internal standard spiking solution. This method verification standard is compared to the concentration verification standard to verify confidence in the pipetting accuracy and technique.

> *Note:* Regularly check the accuracy of repeat pipettes; they can be the source of large errors.

9.8 Unless the same internal standard spiking solution is used for both analytical standards and samples, prepare an internal standard check sample one for every 25 samples. Prepare by pipetting 100 μL intermediate calibration verification solution into 900 μL of the internal standard spiking solution used for analytical samples. The internal standard check sample is used to compare the different internal standard spiking solutions.

9.9 Cap the autosampler vial snugly.

9.10 Refrigerate all samples. Analyze samples as soon as practicable. Do not store analytical samples, prior to analysis, for more then one month.

10. Preparation of Apparatus

10.1 Clean the injection syringe before starting the analysis. Biomass to ethanol fermentation supernatants tend to leave a sticky film on the plunger which affects the precise operation of the syringe.

10.1.1 Remove the plunger from the syringe and wipe down using a paper towel soaked in reagent grade water.

10.1.2 Place a few drops of reagent grade water on top of syringe and force several volumes through with the plunger.

10.2 If possible, utilize any autosampler's self cleaning ability to extensively clean the syringe between injections.

10.3 Change the deactivated glass injection port liner frequently. Typically the isopropanol internal standard area counts begin to fall soon after running fermentation samples. After the first twenty or so analytical sample injections, these values tend to stabilize at a lower sensitivity. It is in this stabilized region that analysis takes place. After approximately 100 additional injections the isopropanol internal standard area counts become unstable and analytical precision is compromised. The injection port liner must be changed at this time.

10.4 Change the GC septum after every 50 injections or so.

11. Calibration and Standardization

11.1 Prepare appropriate concentration internal standard spiking solution, using reagent grade isopropanol. The internal standard spiking solution must be added in the same proportion to every standard or sample analyzed by this method. This procedure specifies nine parts of a 1 g/L internal standard spiking solution be added to one part sample or standard. Therefore, the internal standard concentration is 0.9 g/L universally throughout this procedure.

11.2 Prepare 3 to 6 ethanol analytical standards, using 200 proof ethanol, ranging from 0.1 to 5.0 g/L and all containing 0.9 g/L isopropanol.

11.3 Prepare 1-3 intermediate calibration verification solutions from the separate ethanol source. Dilute 1:10 with internal standard spiking solution, using a volumetric pipette and volumetric flask. When diluted in this manner, the intermediate calibration verification solution becomes a calibration verification standard. The concentration of the calibration verification standard(s) are to be within the range of the analytical standards, but not equal to any of them.

11.3.1 Refrigerate analytical and calibration verification standards in sealed autosampler vials for storage. Properly sealed, the analytical and calibration standards are good for three months.

11.4 Prepare several solvent washes by dispensing reagent grade water into an autosampler vial and seal.

12. Conditions

12.1 Oven temperature: 155°C (isothermal).

12.2 Inlet temperature: 175°C.

12.3 Detector temperature: 250°C.

12.4 Run time: 5.5 minutes.

12.5 Ethanol retention time: About 2.3 minutes.

12.6 Isopropanol retention time: About 4.1 minutes.

12.7 Carrier gas flow rate: 30 ml/min.

12.8 FID flow rates: Per manufacturer's recommendations.

12.9 Injection volume: 1 μl.

13. Procedure

13.1 Warm all standards and samples to room temperature and lightly mix the contents of all the vials.

13.2   Set up analytical run as follows:

13.2.1   Solvent wash.

13.2.2   Analytical standards from low concentration to high.

13.2.3   Calibration verification standard.

13.2.4   Solvent wash.

13.2.5   A batch of samples in randomized order. A batch, typically, is a set of samples from one time point, including associated method verification standards, replicates, and internal standard check standards. A batch usually does not exceed 20 samples.

13.2.6   Calibration verification standard.

13.2.7   Solvent wash.

13.2.8   Repeat 13.2.5-13.2.7, if necessary.

13.3   Recalibrate the standard curve after every 6 hours of analysis.

13.4   Do not reuse analytical or calibration verification standards.

14.   Calculations

14.1   This method utilizes an internal standard which corrects for variations in the injection volume.

14.2   Typically analytical software is utilized to perform concentration calculations. The chromatograms are integrated based upon peak area. The calibration curve is generated by a linear regression of the analytical standards, and ignores the origin as a calibration point. Confirm the linearity of the calibration curve; the coefficient of variation of the linear regression must be greater than 0.99.

14.3   If analytical software is not utilized, the calculation may be done manually. From the standards data, create a calibration curve of amount ratio, plotted on the abscissa, versus response ratio, plotted on the ordinate. The amount ratio is the ethanol concentration divided by the isopropanol internal standard concentration. The response ratio is the ethanol area divided by the isopropanol area.

Calculate a linear regression through the standard points, excluding 0,0 as a data point. The equation of the resulting line should take the form of :

response ratio = *slope (amount ratio)* + *(yintercept)* For the analytical samples, the response ratio is determined and the internal standard concentration is known. Use the equation, above, to solve for the ethanol concentration in the samples.

14.4 The calculated fermentation supernatant concentration needs to account for any dilution that occurred during the preparation of the analytical sample. In this case, multiply the ethanol concentration of the analytical sample by ten.

15. Report

15.1 Report all results to the nearest 0.1 g/L.

16. Precision and Bias and Quality Control

16.1 Analysis in one laboratory of a calibration verification standard at the lower end of the analytical range showed a recovery of 100.32% with a coefficient of variation of 3.60%.

16.2 Analysis in one laboratory of a calibration verification standard at the higher end of the analytical range showed a recovery of 97.93% with a coefficient of variation of 4.67%.

17 Quality Control

17.1 *Reported significant figures:* Report all results to the nearest 0.1 g/L according to section 15.1.

17.2 *Replicates:* Prepare one replicate for every 25 samples prepared according to section 9.6.

17.3 *Blank*: Prepared according to section 11.4. The solvent washes are used check for any carryover problems. There should be no ethanol or isopropanol peaks in the solvent wash runs.

17.4 *Relative percent difference (rpd) criteria*: Calculate the rpd for each duplicate set. Limits for rpd have not been determined precisely, but a rpd greater than 15% is cause for reanalysis.

17.5 *Method verification standard*: Prepared one for every 25 samples prepared according to section 9.7.

17.6 *Calibration verification standard*: Prepared according to section 11.3.

17.7 *Sample size:* 100 µL of filtered fermentation supernatant prepared according to section 9.5.

17.8 *Sample storage*: Refrigerated in autosampler vials for no more than a month according to section 9.10.

17.9 *Standard preparation*: Per section 11.

17.10 *Batch size*: Defined in section 13.2.5.

17.11 *Control Charts*: Calculate the percent recovery for each calibration verification standard run and control chart these values. Control chart the slope of the analytical curve.

17.12 *Matrix effects:* It is assumed that the matrix (fermentation media) for these sample will not vary greatly. If the matrix is considerably changed, it must be shown that the new matrix does not affect the accuracy of this method.